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Gender Is a Major Factor in Determining the Severity of Mycoplasma Respiratory Disease in Mice

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Gender is a significant factor in determining the susceptibility to and severity of pulmonary diseases in both humans and animals. Murine respiratory mycoplasmosis (MRM), due to *Mycoplasma pulmonis* infection, is an excellent animal model for evaluation of the role of various host factors on the development of acute or chronic inflammatory lung diseases. MRM has many similarities to mycoplasma respiratory disease in humans. The purpose of the present study was to determine whether gender has a significant impact on lung disease due to *M. pulmonis* infection in mice. It was demonstrated that male mice consistently developed more severe disease in the lung parenchyma than did female mice. There was no gender difference in disease severity along the airways or any difference in mycoplasma numbers in lungs of male and female mice. Furthermore, surgical removal of reproductive organs reduced the severity of mycoplasma disease and the numbers of mycoplasma organisms recovered from lungs. Thus, gender plays a significant role in determining the severity of *M. pulmonis* disease. In fact, the gender of the host was a major factor in determining whether an acute or chronic inflammatory lung disease developed after infection with *M. pulmonis*.

Respiratory disease is a major health problem in the United States, with males, in general, being more susceptible than females to several major lung diseases (4, 11, 19, 26, 29). Chronic obstructive pulmonary disease, which includes chronic bronchitis, chronic asthma, and emphysema, is the fifth most common cause of death. Chronic obstructive pulmonary disease is especially problematic in the elderly, with males being affected more frequently than females (19). Males are also more likely to develop community-acquired and nosocomial bacterial pneumonias than are females (11, 26, 29). Furthermore, the severity of pneumonia appears greater in male patients, since as males have a higher risk of hospitalization and mortality due to pneumonia (4, 19). In the adolescent patient population, the same tendency exists in that males can also be more susceptible to lung disease than females. This is demonstrated in *Mycoplasma pneumoniae* disease, which is one of the most prevalent respiratory infections in children and young adults (12, 17, 18). Thus, gender has an effect on susceptibility to several pulmonary diseases and may be an unappreciated but significant factor when considering the diagnosis and treatment of respiratory diseases in humans.

Gender also influences the development of infectious disease in animals. Male mice are either more susceptible to or develop more severe disease after infection with *Candida*, coxsackievirus, and *Leishmania* (3, 23, 28). However, there are few animal models of respiratory disease where gender has been shown to influence host susceptibility. After infection with *Mycobacteria marinum* or *Mycobacteria intracellulare*, male mice developed more severe granulomatous lung lesions than did females (39, 40). The difference in disease severity corresponds to the numbers of *M. intracellulare* cells in the lungs. In *M. marinum* infection, as well as the other models of infectious diseases (28, 39), it was further demonstrated that testosterone exacerbated disease severity. Although the results of the studies with mycobacteria are important, lung disease in humans is not limited to the characteristic granulomatous lesions described in these animal models. Therefore, here is a need to establish additional animal models to investigate the influence of gender on respiratory disease.

Murine respiratory mycoplasmosis (MRM) is an excellent animal model for use in evaluation of the role of various factors on the development of acute or chronic inflammatory lung diseases. MRM is a naturally occurring respiratory disease in rodents and results from infection with *Mycoplasma pulmonis* (8, 25, 35). Although it is not an exact model of human disease, there are similarities in the pathology and clinical signs between the mycoplasma respiratory disease in humans and *M. pulmonis* disease in mice. As in many human diseases, host and environmental factors can affect the progression of *M. pulmonis* respiratory disease (13–15, 25, 27, 30, 31). An additional advantage of MRM is that both acute alveolar and chronic peribronchial pneumonias are characteristic of *M. pulmonis* disease in mice. Because of its similarity to human disease and the presence of both acute and chronic inflammation, MRM appears to be an ideal model to examine the effect of gender on the pathogenesis of lung disease. The purpose of the present study was to determine if gender does influence the severity of lung lesions due to *M. pulmonis* infection in mice.

MATERIALS AND METHODS

Animals. Six-week old, specific-pathogen-free C3H/HeN mice, reared and maintained in Trexler-type plastic film isolators, were used in these experiments (30). All retired breeders from the colony were examined for the presence of serum immunoglobulin G (IgG) and IgM antibodies to *M. pulmonis* and *Mycoplasma arthritidis* by enzyme-linked immunosorbent assays (ELISA). The ab-

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sence of other murine pathogens was confirmed using bacterial fecal cultures, necropsy, histological examination, and serologic tests for viruses. Sera from mice were tested by hemagglutination inhibition, complement fixation, or ELISA by Charles River Biotechnical Services (Wilmington, Mass.) for the following pathogens: Sendai virus, pneumonia virus of mice, polyomavirus, minute virus of mice, ectromelia virus, mouse hepatitis virus, reovirus type 3, Theiler's GD-VII virus, lymphocytic choriomeningitis virus, and mouse adenovirus. No murine pathogens have been detected in this animal colony during the past 5 years. Specific-pathogen-free C57BL/6N and DBA/2N mice were obtained from the National Cancer Institute, Frederick Cancer Research Facility, Frederick, Md. Health surveillance was similarly performed on these mice to exclude the presence of murine mycoplasma, viruses, bacteria, and parasites.

Experimental mice were maintained in microisolators with sterile bedding (five to six mice per cage), and sterile food and water was provided ad libitum. Prior to experimental manipulation, mice were anesthetized with a intramuscular injection of 10 mg of ketamine hydrochloride (Bristol Laboratories, Syracuse, N.Y.) per 100 g of body weight and 3.0 mg of xylazine (Haver-Lockhart, Shawnee, Kans.) per 100 g of body weight.

Mycoplasma. *M. pulmonis* strain CT was derived from a naturally infected mouse (14). A defined mixture of mycoplasma subclones, derived from the parental *M. pulmonis* CT strain, was shown to consistently result in both peribronchial and alveolar inflammatory disease (unpublished results). This organism stock is simply referred to as CTM, and *M. pulmonis* CTM was used in these studies unless indicated otherwise. For experimental infection, anesthetized mice were inoculated intranasally with 50 μl of mycoplasma at a total dose of 10^6 CFU, unless otherwise noted. Medium and harvesting techniques were previously described (5, 6, 14).

Lung homogenates were cultured on Hayflick's agar plates to determine the numbers of mycoplasma CFU. Samples were diluted in broth from 10^{-1} to 10^{-6} dilutions. Portions (20 μl) of the diluted broth were pipetted on the agar plates and then incubated for 7 days at 37°C. Mycoplasma colonies were counted using a dissecting microscope.

Surgical removal of reproductive organs. Anesthetized mice were prepared for surgery by shaving the abdomen and swabbing it with Betadine Surgical Scrub (Purdue Frederick, Norwalk, Conn.). A midline surgical incision was made in the abdominal wall. For ovahysterectomy, the uterus, oviducts, and ovaries were removed and ligatures were placed at the uterine-cervical junction. For orchiectomies, the seminal vesicles and testicles were removed. For all surgeries, the abdominal muscular layer was sutured using surgical chromic gut and the skin was sutured with silk. For controls (sham neutered), a midline surgical incision was made on the abdomen and a surgical probe was used by manipulate the reproductive organs. The probe was removed, and the muscular layer and skin were sutured as above. The mice were allowed to recover and observed for 2 to 4 h after surgery. Mice were infected with *Mycoplasma* 2 weeks after surgery.

Histological examination. Lungs and tracheas were removed and inflated with cold 95% ethanol. The lung lobes were separated and placed in tissue cassettes for embedding and subsequent hematoxylin and eosin staining. Each lung lobe was scored subjectively for airway and alveolar disease (6). Airways were scored as follows: 0, normal tissue; 1, few scattered neutrophils in the airways; 2, some pooling of neutrophils in the bronchi and bronchioles; 3, major pooling of neutrophils in the bronchi and bronchioles. Alveolar disease was scored using a similar scale: 0, normal tissue; 1, inflammatory exudate affecting 1 to 25% of the lung parenchyma; 2, inflammatory exudate affecting 26 to 50% of the lung parenchyma; 3, inflammatory exudate affecting more than 50% of the lung parenchyma. Slides were coded so that subjective scoring of lesions was done blindly. Alveolar and airway scores for each lobe were summed after adjustment for their relative contribution to the total lung weight.

Testosterone levels. Serum samples were collected at the time of sacrifice and pooled. Serum testosterone levels were performed using radioimmunoassay (Tri-Level Ligand Control; CIBA-Corning Diagnostics Corp., Irvine, Calif.).

Antibody levels. Anti-mycoplasma antibody titers in the sera were determined using ELISA (6, 34). Serial dilutions of each serum sample were added in triplicate to microtiter wells coated with *M. pulmonis* lysate at 10 μg/ml. After overnight incubation, the plates were washed with phosphate-buffered saline–0.2% Tween 20. A secondary antibody, labeled with alkaline phosphatase, was then added to each of the wells at a dilution shown to have minimal nonspecific binding to antigen-coated wells. Next, the microtiter wells were incubated for 30 min at 37°C, and the optical density was read at 405 nm using a model 3550 microplate reader (Bio-Rad Laboratories, Richmond, Calif.). The relative antibody activities were determined relative to a standard composed of a pool of high-titer sera from *M. pulmonis*-infected mice. The standard was given an arbitrary activity of 3,000 and used to compare the activity of other sera. Comparisons between serum activities were made only with values obtained within a single ELISA run to eliminate daily variation within the assay.

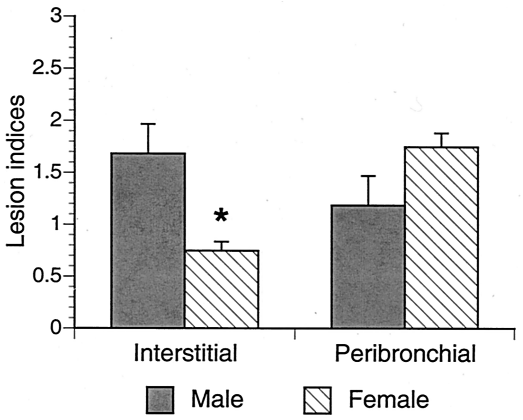

FIG. 1. Lung disease in male and female mice after infection with *M. pulmonis*. The graph shows the mean lesion index scores, along with standard error bars, from male and female mice infected with *M. pulmonis* 14 days previously. No significant difference was observed in the severity of peribronchial lesions between male and female mice. However, there was a significant difference ($P \leq 0.05$) in the severity of interstitial lung lesion between the sexes. The asterisk denotes a significant difference in lesion indices between male and female mice.

Statistical analysis. Statistics were performed using the SYSTAT program (Systat, Inc., Evanston, Ill.). Arcsine transformation was performed on lesion indices to normalize the data. Data were analyzed by analysis of variance followed by post hoc tests for multigroup comparisons, as needed. Survival and frequency of gross lesions were compared by Yates corrected chi-square analysis. Testosterone levels were analyzed by Student's independent *t* tests. A probability (*P*) of less than 0.05 was accepted as significant.

RESULTS

Male mice develop more severe alveolar pneumonia than female mice do. To determine if gender influences the severity of *M. pulmonis* respiratory disease, male and female mice were experimentally infected with *M. pulmonis*. Animals were observed daily for clinical signs and mortality. At 14 days after infection, mice were sacrificed and their lungs were collected for histologic examination.

Male mice infected with *M. pulmonis* developed more severe clinical disease than did female mice. The clinical signs seen included ruffled fur, weight loss, and a hunched position. Female mice were consistently more active than male mice, and those that survived even showed signs of improvement. Overall, a larger proportion of male mice than female mice died after infection ($P \leq 0.001$). In three experiments, 60% of male mice died ($n = 22$) compared to only 9% of the female mice ($n = 33$).

There was also a histopathological difference in the lungs of male and female mice inoculated with *M. pulmonis* (Fig. 1). Lung lesions in male mice were associated predominantly with an alveolar disease characterized by thickened alveolar walls, edema, hemorrhage, and accumulation of acute inflammatory cells. In addition, the airways and adjacent air spaces contained inflammatory exudates. The character of the lesions in the male mice was the same whether the mice survived or not. In contrast to male mice, alveolar disease was minimal in female mice but the severity of airway lesions was similar to that in male mice.

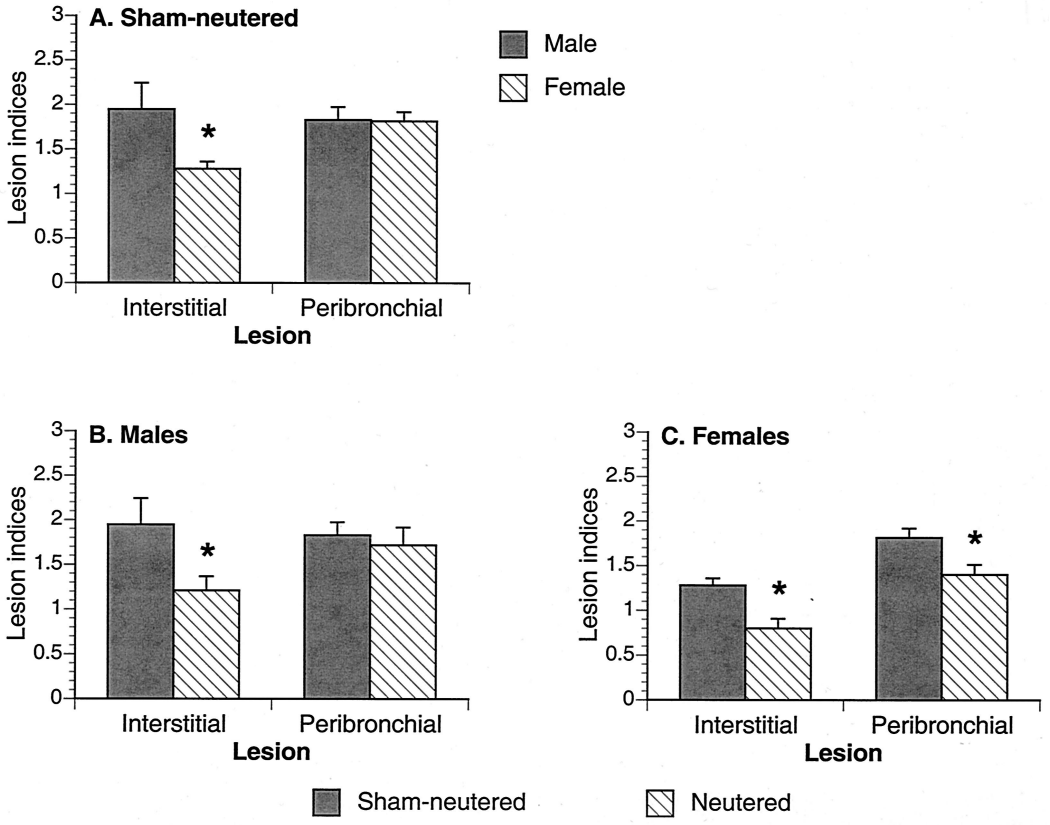

FIG. 2. Effect of neutering on the severity of *M. pulmonis* disease at 14 days after infection. Neutered male and female mice were infected with *M. pulmonis*, and the severity of interstitial and peribronchial lesions was determined 14 days after infection. Sham-neutered mice were included for comparison. (A) As in earlier studies, no significant difference was observed in the severity of peribronchial lesions between sham-neutered male ($n = 12$) and female ($n = 11$) mice. However, there was a significant difference ($P \leq 0.05$) in the severity of the interstitial lung lesion between the sexes. (B and C) Removal of reproductive organs from male ($n = 8$) and (B) female ($n = 7$) (C) mice significantly decreased ($P \leq 0.05$) the severity of disease. Removal of the reproductive organs of infected male mice resulted in a significant decrease in alveolar inflammation, while removal of the reproductive organs of female mice led to a decrease in the severity of both interstitial and peribronchial lesions. Gonadectomy had no significant effect on the peribronchial inflammatory response. The graph shows the mean lesion index scores, along with standard error bars, from lungs of infected mice. The asterisk denotes a significant difference in lesion indices between male and female mice (A) or reduction in severity after surgery (B and C).

Neutering of mice reduces the severity of mycoplasma lung disease. To examine the influence of the reproduction organs on the development of lesions after infection, male and female mice were neutered at 6 weeks of age. Sham-neutered mice were included as controls. The mice were infected with *M. pulmonis* after 14 days of recovery and then sacrificed at 14 days after infection, and their lungs were removed for histological examination.

Removal of the reproductive organs reduced the severity of lung lesions in both male and female infected mice. As in previous experiments, infected male mice developed an acute interstitial pneumonia while infected female mice developed a chronic bronchopneumonia (Fig. 2). Sham-neutered male mice infected with *M. pulmonis* had more severe interstitial lung disease than did neutered male mice. In neutered male mice, the severity of inflammatory cell infiltrates was reduced. Similarly, there was a reduction in the severity of the peribronchial lesions in female mice after surgical removal of the reproductive organs. There were no observable histological changes in the lungs of uninfected male and female mice after surgery.

Gender differences were also present earlier than 14 days. For mice infected with *M. pulmonis* and sacrificed at 7 days postinfection, male mice had an acute inflammatory disease of the alveoli while the disease in female mice was localized to the airways of the lungs. In both sexes, pulmonary disease was less severe in mice that had undergone gonadectomy (Fig. 3).

Orchiectomies decreased the presurgical levels of testosterone in male mice. The mean level of testosterone in males before surgery was 842 ± 217 ng/ml (mean ± standard error). Postsurgery, the level of testosterone was significantly reduced to 67 ± 43 ng/ml ($P \leq 0.05$).

Influence of gender and neutering on mycoplasma numbers in the lung. To determine if the effects of gender and surgery were associated with a change in the numbers of mycoplasma in the lungs, neutered and sham-neutered mice were infected at 8 weeks old and sacrificed at 7 days postinfection. There was no significant difference in the numbers of mycoplasma recovered from the lungs of sham-neutered male and female mice after infection (Table 1). However, surgical removal of reproductive organs affected the numbers of mycoplasmas in lungs. At 7 days after infection, neutered male mice had significantly

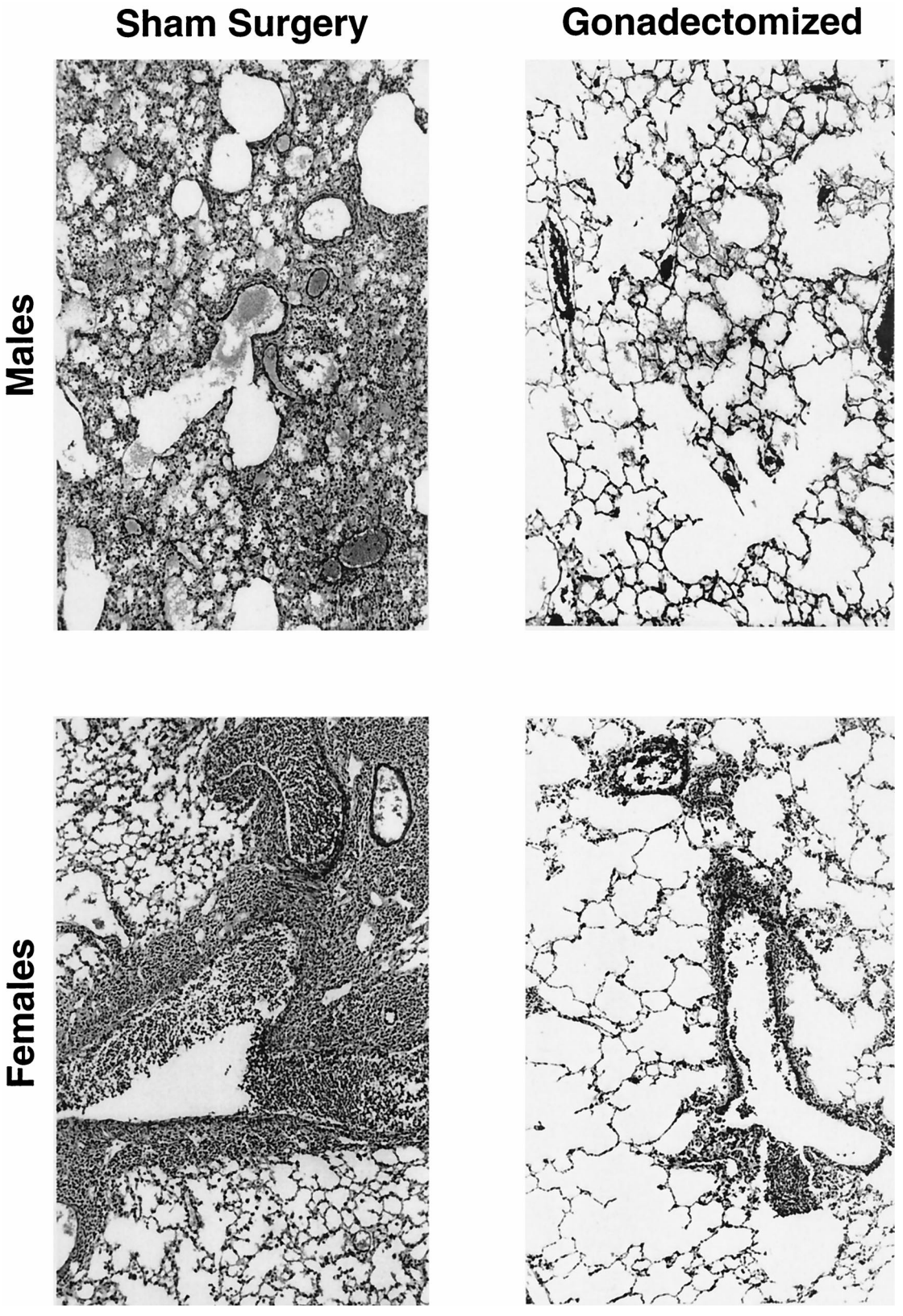

FIG. 3. Effect of neutering on *M. pulmonis* disease in mice at 7 days after infection. Neutered male and female mice were infected with *M. pulmonis*, and the severity of interstitial and peribronchial lesions was determined 7 days after infection. Sham-neutered mice were included for comparison. These are representative sections from each of the experimental groups. Sham-neutered male mice ($n = 11$) had more severe inflammatory response in the alveoli than did neutered male mice ($n = 13$). Similarly, sham-neutered female mice ($n = 15$) had more severe lung lesions than did female mice with ovohysterectomies ($n = 15$).

TABLE 1. Influence of neutering on mycoplasma numbers in the lungs

Gender	Control mice		Neutered mice	
	CFU[a]	*n*	CFU[a]	*n*
Male	59×10^3 (1.4)	11	17×10^3 (1.7)[b]	10
Female	33×10^3 (1.5)	11	4.4×10^3 (1.2)[b]	12

[a] Geometric mean (x/÷ standard error) mycoplasma numbers recovered from the lungs at 7 days postinfection.
[b] Neutered male and female mice had a significantly smaller number of mycoplasmas in their lungs than did sham-neutered mice ($P \leq 0.05$). There was no significant difference between the mycoplasma numbers recovered from sham-neutered male and female mice.

smaller numbers of mycoplasmas in lungs than did control (sham-neutered) male mice. Similarly, smaller numbers of mycoplasmas were recovered from the lungs of neutered female mice than of sham-neutered female mice after infection. Lastly, there was a significant difference in the mycoplasma numbers obtained from infected male mice that were neutered and from infected female mice that were neutered.

Serum anti-mycoplasma antibody levels in mice. We determined the serum antibody levels of surgically neutered mice and sham-neutered mice infected with *M. pulmonis* at 7 days postinfection. Only very low levels of *M. pulmonis*-specific IgA and IgG were detected, although significant levels of *M. pulmonis*-specific IgM were present. Sham-neutered male mice had an IgM titer of 555 ± 57, compared 570 ± 75 for neutered male mice. The mean IgM titers were 288 ± 57) and 438 ± 57 for sham-neutered and neutered female mice, respectively. Overall, there was no significant difference in the IgM antibody response between male and female mice in any of the experimental groups.

Gender influences the severity of mycoplasma disease in other mouse strains. To determine if gender influences the severity of *M. pulmonis* respiratory disease in other strains of mice, C57BL/6N and DBA/2N mice were infected with 2×10^4 CFU of *M. pulmonis* CT and mortality and the presence of gross lung lesions were noted at 21 days after infection. Overall significantly greater numbers of C57BL/6N male mice had gross lung lesions than C57BL/6N female mice (Table 2). Also, there was a higher mortality in DBA/2N male mice than DBA/2N female mice.

DISCUSSION

Although gender is a significant factor that affects the susceptibility to and severity of respiratory disease in humans, there are few animal models of infectious lung disease where gender is shown to influence the outcome. MRM due to *M. pulmonis* infection in mice is an excellent animal model of acute and chronic inflammatory lung disease (7, 9). The purpose of the present studies was to determine if *M. pulmonis* disease in mice is affected by gender.

Male mice infected with *M. pulmonis* developed more severe clinical disease and had higher mortality than did infected female mice. Female C3H mice displayed a chronic wasting syndrome characterized by weight loss, ruffled fur, and hunched appearance but with few deaths. However, male C3H mice often exhibited a fatal shock-like syndrome within 4 to 6 days after infection. Gender differences were also present in DBA/2N and C57BL/6N mice infected with *M. pulmonis*. Thus, the gender differences in disease susceptibility were not unique to one strain of mice, indicating that the increased susceptibility of male mice to severe mycoplasma respiratory disease is a common phenomenon.

Pulmonary histopathological tests demonstrates that gender affects the type and character of the inflammatory response in C3H mice. In *M. pulmonis*-infected male mice, the pulmonary lesion was an acute inflammatory response in the alveoli, characterized by a predominantly neutrophilic infiltrate, edema, and hemorrhage. In contrast, infected female mice developed a chronic peribronchial inflammatory response with an infiltration of mononuclear cells and few neutrophils. This difference in the inflammatory response between the sexes was not due to the number of organisms, because the mycoplasma numbers in the lungs of male and female mice were similar. Thus, the difference due to gender in mycoplasma disease was independent of the clearance of the organism but was related to the mechanisms which regulate the type and character of the inflammatory response.

Sex hormones appear to directly or indirectly influence the inflammatory process and host resistance to mycoplasma in the lungs. *M. pulmonis*-infected male mice which were neutered developed less severe lung lesions and had correspondingly smaller numbers of mycoplasma in the lungs than did infected control mice. A similar decrease in disease and infection was observed in female mice after neutering. Surgical removal of male gonads has been reported to increase protection against other infections (28, 39). For example, gonadectomized BALB/c male mice infected with *M. marium* had increased host resistance, smaller numbers of organisms, and less severe lung lesions than did infected control males (39). In preliminary studies, we also found that testosterone treatment of neutered female mice increased the severity of lung disease due to *M. pulmonis* (unpublished results). Although sex hormones are probably involved in mycoplasma disease, the mechanisms responsible for the gender differences in inflammatory lesions, independent of the clearance of the organism, are unknown.

Since the severity of inflammation is regulated by lymphocyte and macrophage activities, gender differences may be linked to one or both of these cell populations. In previous studies, we demonstrated that lymphocyte activity has both beneficial and deterimental effects on the pathogenesis of mycoplasma disease, including the development of pulmonary inflammation (5). In support of lymphocyte responses contributing to the gender differences in mycoplasma disease, female mice are

TABLE 2. Gender differences after infection with *M. pulmonis*[a]

Outcome of infection	% of mice showing outcome[b]			
	C57BL/6		DBA/2N	
	Female	Male	Female	Male
Gross lung lesions	0 (0/15)	53 (8/15)[c]	100 (16/16)	100 (16/16)
Death	0 (0/15)	0 (0/15)	38 (6/16)	100 (16/16)[c]

[a] C57BL/6N and DBA/2N mice were infected with 2×10^4 CFU *M. pulmonis* CT. Mortality and the presence of gross lung lesions were noted 21 days postinfection.
[b] Percentage of affected mice (number of affected mice/total number of mice).
[c] A significant difference between male and female mice was found ($P \leq 0.05$). Experiments were performed twice.

known to develop higher antibody responses to various antigens after immunization (1, 33). Also, sex hormones regulate T-cell-mediated immune function (24, 37, 38). However, we found no significant difference in the levels of anti-*M. pulmonis* antibody in serum in male and female mice at 7 days after infection. At this time point, the predominant antibody response was of the IgM class, with low levels of IgG and IgA. Differences in disease due to gender were observed within the first week after infection, further suggesting that adaptive immune responses are not responsible for these phenomena. In support, preliminary studies demonstrate that these gender differences are observed in mice with severe combined immunodeficiency (SCID mice), which lack functional T and B cells (unpublished results). In addition, sex hormones are able to modulate the activation of macrophages, including the production of proinflammatory cytokines (2, 32, 36). Thus, the differences in the early stage of *M. pulmonis* disease in male and female mice may be due to the proinflammatory activity of macrophages in the lungs (2, 32, 36) rather than to gender differences in their anti-mycoplasmal activity in the lungs (10, 16, 20–22, 36, 39, 40). However, additional work is needed before we can further understand the mechanisms through which gender affects the susceptibility to respiratory diseases.

In summary, this is the first study to demonstrate that gender does play a significant role in determining the severity of mycoplasma respiratory disease in mice. In fact, the gender of the host was a major factor in determining whether an acute or chronic inflammatory lung disease developed after infection with *M. pulmonis*. This is in contrast to the gender differences described for mycobacterial infection of mice, where only granulomatous lung lesions are present (39, 40). The results of these studies also suggest that modulation of innate host defenses and proinflammatory responses by sex hormones is a major factor in these gender-related differences. Although gender is a significant factor in lung disease in humans and animals, the mechanisms for these differences are not clearly understood; however, mycoplasma disease in mice will serve as an excellent animal model to further delineate the role of sex hormones on both acute and chronic inflammatory lung diseases.

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